Postharvest Biology and Technology 145 (2018) 108–116

Contents lists available at ScienceDirect

Postharvest Biology and Technology

journal homepage: www.elsevier.com/locate/postharvbio

Impact of high CO_2 levels on heat shock proteins during postharvest storage of table grapes at low temperature. Functional *in vitro* characterization of VVIHSP18.1

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ARTICLE INFO

Keywords:
Heat shock proteins
Vitis vinifera
Carbon dioxide
Low temperature
Chaperone activity

ABSTRACT

The role played by heat shock proteins (HSPs) in improving fruit quality during postharvest treatments has mainly been studied regarding heat treatments, while little is known about the effect of CO_2 treatments. In this study, we have analyzed the gene expression of five heat shock proteins (*HSPs*) and one heat shock factor (*HSF*) in the skin of red table grapes (*Vitis vinifera* cv. Cardinal) to determine whether a pretreatment with high CO_2 levels (20 kPa) modulated their expression and how the length of the treatment (1 or 3 d) could influence this change. The 3-d high CO_2-treatment was effective in reducing total decay and induced the accumulation of three small HSPs (*VviHSP18.1*, *VviHSP18.2* and *VviHSP22.0*), whereas *VviHSP70.0* and *VviHSF4-a* gene expression were induced by both treatments. To shed light on the putative physiological role of a small HSP (VviHSP18.1) acting as a chaperone, the recombinant protein was overexpressed in *Escherichia coli*. It was then purified and mass spectrometry confirmed that the isolated protein was VviHSP18.1, belonging to class I sHSP. Although the purified protein was stable at different high temperatures, when temperature was above 70 °C, a weaker and smaller protein band appeared which was identified by mass spectrometry as VviHSP18.1 with a C-terminal truncation. The recombinant VviHSP18.1 protein displayed chaperone activity which protects citrate synthase (CS) and malate dehydrogenase (MDH) from thermal aggregation at 45 °C, and also displayed the protection of alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH) activities at 55 °C and 65 °C, respectively. By contrast, VviHSP18.1 did not protect LDH from freezing-induced inactivation. Taken together, these results support the hypothesis that a high CO_2 treatment is an active process where HSPs could participate in preventing the denaturation and dysfunction of different proteins.

1. Introduction

Plants are sessile organisms which have developed complex mechanisms for perceiving environmental biotic and abiotic stresses, and respond by altering the expression of defense-signaling molecules. Since exposure to different stresses normally causes protein dysfunction, it is particularly important for plants to maintain their functional conformations and prevent the aggregation of non-native proteins in order to maintain cellular homeostasis under stressful conditions. Plants have specific proteins for this purpose, among which is one particular family that comprises heat shock proteins (HSPs) (reviewed by Aghdam et al., 2015). While HSPs were first described in relation to high temperatures, they are also induced in plants in response to different abiotic stresses, such as salinity, osmotic, cold and water stress (Wang et al., 2004). Five main HSP families have been identified in plants and grouped according to their average molecular weights: Hsp100s, Hsp90s, Hsp70s, the chaperonins Hsp60s and the small HSPs (sHsp) family. Transcription of HSP genes is regulated by heat shock transcription factors (HSFs) through binding to *cis*-acting regulatory elements called heat shock element (HSEs), located in the promoter region of HSP genes (Scharf et al., 2012).

It has previously been suggested that HSPs exert their protection during stress mainly due to their biological function as molecular chaperones which prevent protein aggregation and promote the proper refolding of denatured proteins, as well as assisting in the assembly and disassembly of protein complexes and in protein degradation (Hartl and

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https://doi.org/10.1016/j.postharvbio.2018.06.006
Received 14 March 2018; Received in revised form 4 June 2018; Accepted 27 June 2018

Hayer-Hartl, 2002; Kim et al., 2007; Ezemaduka et al., 2017). Among the molecular chaperone families, sHSP are the only known ATP-independent chaperones (Jakob et al., 1993) and as "holdase" chaperones (though incapable of folding polypeptides *per se*), they protect cells from protein aggregation. However, they must release their aggregation-prone clients to other downstream chaperones, which then ensure the correct folding (Eyles and Gierasch, 2010).

Sanchez-Bel et al. (2012) suggested that the accumulation of sHSP is key as the first response of tomato fruit to chilling injury. Recently, Ré et al. (2017) reported the increased expression of *sHSPs* in Micro-Tom fruit stored at 4 °C, substantiating the hypothesis that sHPSs may participate in the mechanisms involved in the protection against cold stress. It is known that HSPs not only protect against the stress that causes their accumulation but also against any subsequent stressful situation (Lin et al., 1984; Chen et al., 1988). The induction of *HSPs* gene expression after a moderately high temperature (6 h–72 h at 38 °C) in both avocado (Woolf et al., 1995) and tomato (Sabehat et al., 1998) fruit has been shown to protect them during subsequent prolonged low temperature storage and was correlated with protection against chilling injury. Likewise, in a recent work Salazar-Salas et al. (2017) indicated that hot water at 42 °C for 5 min induced chilling injury tolerance in tomato fruit and up-regulation and greater protein accumulation of sHSP during cold storage and after ripening. These findings are associated with cross-tolerance phenomena in which plants exposed to one type of stress condition may gain protection against other biotic or abiotic stresses (Bowler and Fluhr, 2000; Pastori and Foyer, 2002; Wang et al., 2003). Aghdam et al. (2013) indicated that various postharvest treatments such as chemical ones (treatments with salicylate and derivatives) and physical ones (hot water/hot air pretreatments, preconditioning at medium-low temperatures, UV-C light) applied to reduce chilling injury during storage of different fruit triggered *HSP* gene expression and protein accumulation.

In previous studies, we observed that applying a short-term high $-CO_2$ treatment (20 kPa CO_2) for 3 d at low temperature maintained the quality of different table grape cultivars during postharvest storage and improved the tolerance to temperature shift at 0 °C (Sanchez-Ballesta et al., 2007; Rosales et al., 2016; Vazquez-Hernandez et al., 2017). In a recent transcriptional analysis, we found that genes coding for HSPs were strongly represented among those up-regulated at 0 °C in the skin of non-treated Cardinal table grapes (Rosales et al., 2016). However, it is interesting to note that a small number of *HSPs* and *HSFs*, which are different from those activated in non-treated grapes, were induced by high CO_2 levels. To date, information about modulation of HSPs gene expression in *Vitis vinifera* is restricted to high temperatures (Liu et al., 2012; Carbonell-Bejerano et al., 2013; Wu et al., 2015), high light (Carvalho et al., 2011) or UV-C irradiation (Xi et al., 2014), whereas the effect of high CO_2 levels remains unknown. To our knowledge, the only study in *V. vinifera* with high CO_2 levels was carried out using detached wine grapes (cv. Trebbian) treated with 30 kPa of CO_2 for 3 d at 20 °C, which was effective in altering the general metabolism and inducing the expression of a *sHSP* (Becatti et al., 2010). In order to find out whether the application of high CO_2 levels modulate the expression of *HSPs* and *HSFs* in table grapes and also whether the length of the treatment affects this modulation, we have analyzed the gene expression of five *HSPs* (*VviHSP18.1*, *VviHSP18.2*, *VviHSP22.0*, *VviHSP70.0* and *VviHSP90.1*) and *VviHSF4-a* in the skin of Cardinal table grapes treated with high CO_2 for 1 d and 3 d during postharvest storage at 0 °C. Furthermore, to investigate the behavior of *V. vinifera* sHSPs, we produced the recombinant VviHSP18.1 protein in *Escherichia coli*. The purified recombinant VviHSP18.1 was stable at high temperature and remained soluble at 45 °C, although temperatures above 70 °C affected the stability since a putative truncation at the C-terminal seems to occur. Likewise, our results showed that the recombinant VviHSP18.1 behaves, *in vitro,* as a molecular chaperone which protects enzyme from thermal aggregation and thermal inactivation at high temperatures. The results obtained in this study could lead to a more thorough characterization of the molecular basis implicated in the beneficial effect of high CO_2 levels in maintaining table grape quality.

2. Materials and methods

2.1. Plant material

Table grapes (*Vitis vinifera* cv. Cardinal) were randomly collected in Camas (Sevilla, Spain) at the early commercial stage (15.73% soluble solids, 0.38% tartaric acid). The clusters were transferred to the ICTAN laboratory in Madrid (Spain) on the same day of harvesting and fruit were immediately forced-air precooled for 14 h at −1 °C. Thereafter, those that did not present mechanical or pathological defects were randomly divided into three lots and stored at 0 ± 0.5 °C with a relative humidity of 95% in three sealed neoprene containers of 1 m^3 capacity. Ten boxes with a content of around 3 kg of table grapes per box were stored in each container. One lot was stored under normal atmospheric conditions for a total of 13 d (non-treated fruit). The other lot was stored with a gas mixture containing 20 kPa CO_2 + 20 kPa O_2 + 60 kPa N_2 for 3 d (3-d CO_2-treated fruit) and then transferred to air under the same conditions as the non-treated bunches until the end of the storage period. In order to analyze the effectiveness of the treatment with high CO_2 levels during short time, a third lot of bunches was stored with high CO_2 levels for 1 d (1-d CO_2-treated fruit), then transferring the clusters to normal atmospheric conditions until the end of the storage period. At time 0 (after precooling at −1 °C) and after 1, 3, 6 and 13 d of storage under air or CO_2 conditions, berries from three biological replicates (each replicate consisting of two bunches) were peeled and the skin was collected, frozen in liquid nitrogen, grounded to a fine powder, and stored at −80 °C until analysis.

2.2. Quality assessments

SSC (expressed in %) was determined using a digital refractometer (Atago PR-101, Japan) at 20 °C. TA (expressed in % tartaric acid) was determined by titration with 0.1 N NaOH up to pH 8.1. Total decay was assessed as the percentage of decayed berries with respect to the original cluster weight.

2.3. RNA extraction, cDNA synthesis

Total RNA extraction and cDNA synthesis was performed according to Romero et al. (2016).

2.4. Isolation and purification of partial HSPs

The oligonucleotides used to determine the expression pattern of the different *VviHSPs* (*VviHSP18.1*, *VviHSP18.2*, *VviHSP22.0*, *VviHSP70.0* and *VviHSP90.1*) and the transcription factor *VviHSFA4-a* were designed using the program Primer 3 (Untergasser et al., 2012) from the information of the sequences used to design the GeneChip® oligonucleotide array of Affymetrix used in previous studies (Rosales et al., 2016) (see Table S1). The specificity of the oligonucleotides was checked by PCR (95 °C 4 min, 91 °C 1 min, 57 °C 1 min, 72 °C 1 min, 72 °C 10 min) using a mixture of cDNAs from treated and non-treated samples with CO_2 based on the results of the transcriptomic analysis of the mechanisms of adaptation to high concentrations of CO_2 applied during 1 or 3 d in table grape (data not shown). The PCR fragments were analyzed on a 2% agarose gel and the fragments were purified using the "Clean-Easy Agarose Purification" kit (Canvax) following the manufacturer's instructions. The sequence of the partial *HSPs* and *HSF* was confirmed by Sanger sequencing at the Genomics Department of the Biological Research Center (CIB-CSIC, Madrid, Spain).